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(54) Title: METHODS FOR DETECTION OF CRYPTOSPORIDIUM OOCYSTS

(57) Abstract

A method of detecting the presence of viable *Cryptosporidium* oocysts in a sample containing *Cryptosporidium* oocysts, the method comprising the steps of a) treating the sample so as to cause any viable oocysts of *Cryptosporidium* in the sample to excyst, b) exposing the treated sample to an antibody that binds specifically to recently excysted *Cryptosporidium* oocysts such that the antibody binds to recently excysted *Cryptosporidium* oocysts in the sample, and c) detecting the presence of oocyst-bound antibody in the sample.

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Methods for Detection of *Cryptosporidium* Oocysts

Technical Field

The present invention relates to antibodies reactive to recently excysted oocysts of *Cryptosporidium* and methods of detecting viable *Cryptosporidium* oocysts using the antibodies.

Background Art

The protozoan parasite *Cryptosporidium* is amongst the most common pathogens responsible for diarrhoeal disease in humans (Current 1986). Infection occurs when *Cryptosporidium* oocysts shed in the faeces of infected individuals are ingested by new hosts. Recently, several large outbreaks of cryptosporidiosis have occurred in which drinking water has been identified as the source of infection (Smith and Rose 1990, Badenoch 1990). Surveys have shown that many surface water supplies are contaminated with *Cryptosporidium* oocysts (LeChevallier *et al* 1991, Rose 1988).

The detection of *Cryptosporidium* oocysts in water relies on the concentration of particulate matter including oocysts from large volumes of water prior to staining with fluorescently labelled antibodies. Until recently, detection and identification of fluorescently labelled oocysts required examination of the sample using epifluorescence microscopy. The tedious and labour intensive nature of this detection method, in particular the amount of fluorescent microscopy required, limited the monitoring work which could be performed. The development of flow cytometric detection methods has alleviated many of these problems and enabled the routine monitoring of water for the presence of *Cryptosporidium* oocysts (Vesey *et al* 1994). A major limitation of this methodology, however, is the lack of oocyst viability measurements.

A further problem with the currently employed method for flow cytometric analysis of water samples for *Cryptosporidium* is the requirement of a flow cytometer that can sort particles for subsequent microscopical examination. Sorting flow cytometers are expensive and sophisticated instruments that require a highly skilled operator.

The flow cytometric method involves staining of samples with a fluorescently labelled monoclonal antibody specific to the surface of *Cryptosporidium* oocysts and then analysis with a sorter flow cytometer. Particles with the fluorescence and light scatter characteristics of *Cryptosporidium* oocysts are sorted onto a microscope slide and examined

manually using epifluorescence microscopy to confirm their identity as oocysts. This confirmation step is necessary because the cytometer is unable to distinguish oocysts from all other particles present in water samples. The particles that the cytometer can mistake as oocysts are autofluorescent particles such as algae or particles that bind the oocyst-specific antibody.

Analysis flow cytometers are available which are simple to operate and relatively inexpensive. These cytometers, however, are unable to perform sorting. To enable the detection of *Cryptosporidium* oocysts using one of these analysis cytometers the discrimination achieved by the cytometer must be improved so that non-oocyst particles are not mistaken as oocysts. The present inventors have shown previously that it is possible to detect a single specific microorganism in turbid water samples with an analysis cytometer (Vesey *et al* 1994B) if the microorganism is labelled with two different antibodies.

Korich et al (1993) reported on the use of a monoclonal antibody for assessing *Cryptosporidium* oocyst viability. The antibody recognises an epitope on the inside of the oocyst and the antibody will only bind to an oocyst after being ruptured. The method described the use of the antibody to determine if oocysts have a ruptured wall. An oocyst with a ruptured wall, however, will not be viable and excysted oocysts are not the same as ruptured oocysts. The antibody therefore is not particularly suitable to detect viable oocysts.

The presence of non-viable *Cryptosporidium* oocysts in drinking water is of little significance to public health. If oocysts are viable, however, the risk to public health is enormous. There is therefore a real need to develop an effective method for determining the viability of *Cryptosporidium* oocysts in water. Furthermore, a method for assessing oocyst viability that is applicable to flow cytometry would enable the technique to be applied to the routine monitoring of water.

Disclosure of the Invention

In a first aspect, the present invention consists in a method of detecting the presence of viable *Cryptosporidium* oocysts in a sample containing *Cryptosporidium* oocysts, the method comprising the steps of:

a) treating the sample so as to cause any viable oocysts of *Cryptosporidium* in the sample to excyst;

b) exposing the treated sample to an antibody that binds specifically to recently excysted *Cryptosporidium* oocysts such that the antibody binds to recently excysted *Cryptosporidium* oocysts in the sample; and

c) detecting the presence of oocyst-bound antibody in the sample.

It will be appreciated that any treatment of the sample that causes oocysts to undergo excystation would be suitable. It is, however, presently preferred that the oocysts are caused to excyst by incubating the sample at about 37°C under acidic conditions, followed by incubating the sample under neutral to alkaline conditions at about 37°C. More preferably, the oocysts are caused to excyst by incubating the sample at 37°C at pH 2 to 4 for 10 to 60 minutes, followed by incubating the sample at 37°C at pH 7 to 9 for 10 to 60 minutes. The sample can be washed between the steps to facilitate the removal of the buffers and replacement with fresh buffers at the required pH.

Recently excysted *Cryptosporidium* oocysts are defined as oocysts that have excysted within several hours from treatment. Usually, the oocysts are exposed to antibody within one hour or less from being excysted to ensure optimal binding. It has been found that the antigen or antigens present on recently excysted oocysts to which antibodies can be made do not remain intact over prolonged periods. It will be appreciated that this period can be increased if the treated sample or oocysts are preserved in some manner. For example, freezing of the sample has been found to preserve the antigenicity of the excysted oocyst past this several hour period.

The present inventors have found that short-lived antigens are present on recently excysted *Cryptosporidium* oocysts and that specific antibodies can be raised against these antigens. These antibodies can be used to detect viable *Cryptosporidium* oocysts in samples. It will be appreciated that by following the teaching of the present invention useful antibodies can be produced against recently excysted *Cryptosporidium* oocysts.

In a preferred embodiment of the first aspect of the present invention, the sample is analysed by flow cytometry or microscopy to detect the oocyst-bound antibody. The binding of the antibody to the recently excysted oocysts can be measured indirectly by further treating the sample with a fluorescently-labelled ligand that binds specifically to the antibody and measuring the binding of the labelled ligand to the oocyte-bound antibody. Alternatively, the antibody can be fluorescently labelled prior to use and the binding of the

antibody to the recently excysted oocysts can be detected by measuring directly the fluorescence of the bound antibody.

In a further preferred embodiment of the first aspect of the present invention, the antibody is a monoclonal antibody, more preferably the monoclonal antibody is Cry4, Cry5 or Cry6, and most preferably Cry4.

In another preferred form of the method of the present invention, the treated oocysts are exposed to a first antibody that binds specifically to recently excysted oocysts and a second antibody that binds specifically to the surface of *Cryptosporidium* oocysts. Preferably the first and second antibodies are labelled with different fluorescent markers such that antibody binding can be detected by measuring the respective fluorescence of each fluorescent marker with a simple analysis-only flow cytometer.

The second antibody preferably binds to both viable and non-viable oocysts and the detection of the binding of one or more of the antibodies to the oocysts can be used to indicate the presence *Cryptosporidium* oocysts in the sample. In a preferred form, the first antibody is Cry4, Cry5 or Cry6, preferably Cry4, and the second antibody is Cry26. When the two antibodies are used as described above, it is possible to differentiate between viable and non-viable oocysts in a sample with the one test.

The method of the present invention is particularly suitable for the detection of viable *Cryptosporidium parvum* oocysts.

In a second aspect, the present invention consists in an antibody that binds specifically to recently excysted oocysts of *Cryptosporidium*. Preferably, the antibody is a monoclonal antibody and more preferably the monoclonal antibody is Cry4, Cry5 or Cry6.

In a third aspect, the present invention consists in an hybridoma cell producing a monoclonal antibody that binds specifically to recently excysted oocysts of *Cryptosporidium*. More preferably the hybridoma cell produces the monoclonal antibody Cry4, Cry5 or Cry6.

In a fourth aspect, the present invention consists in a ligand or ligands of recently excysted oocysts of *Cryptosporidium* that is specifically bound by the monoclonal antibody Cry4, Cry5 or Cry6.

In order that the nature of the present invention may be more clearly understood, preferred forms thereof will be described with reference to the following examples and drawings.

Brief Description of the Drawings

Figure 1 shows flow cytometric analysis of pure excysted oocysts labelled with monoclonal antibodies;

Figure 2 shows flow cytometric analysis of pure non-excysted oocysts reacted with monoclonal antibodies;

Figure 3 shows flow cytometric analysis of pure excysted oocysts stained with Cry4; and

Figure 4 shows flow cytometric analysis of environmental samples seeded with excysted oocysts and treated with two antibodies Cry26 and Cry4;

Modes for Carrying Out the Invention

MATERIALS AND METHODS

***Cryptosporidium* oocysts.** *Cryptosporidium parvum* oocysts cultured in lambs and purified by density gradient centrifugation were purchased from the Moredun Animal Research Institute, Edinburgh.

Monoclonal antibodies. Two female balbC mice were injected with oocyst preparations as presented in Table 1.. Mice were sacrificed, spleen cells dissected and fused with NS1 mouse myeloma cells and the resulting hybridomas cloned. Clones were screened for anti-oocyst antibody production by screening against fresh and excysted oocyst preparations with flow cytometry.

Excysted oocysts were prepared by excysting oocysts and then washing in saline solution.

Excysted SDS treated oocysts were prepared by excysting oocysts, treating with 1% (w/v) sodium deoxycholate at 21°C for 10 minutes and then washing in saline solution.

Table 1. Description of inoculation program.

Mouse 1			Mouse 2		
Days	Antigen	Route	Days	Antigen	Route
0	1.5×10^6 sonicated oocysts in FCA	I.P.	0	1×10^6 sonicated oocysts in FCA	I.P.
33	0.3×10^6 sonicated oocysts in FIA	I.P.	53	9.1×10^6 excysted oocysts in FIA	I.P.
82	1×10^6 heat killed (80°C for 10 minutes) oocysts	I.P.	83	3.1×10^6 excysted SDS treated oocysts in FIA	I.P.
103	1×10^6 sonicated oocysts in FIA	I.P.	225	3×10^6 gamma irradiated oocysts in FIA	I.P.
121	1×10^6 sonicated oocysts in saline	I.V.	259	5×10^6 gamma irradiated oocysts in saline solution	I.V.
121	4×10^6 sonicated oocysts in saline	I.P	281	2.5×10^6 gamma irradiated oocysts in saline solution	I.P.
			282	2.5×10^6 gamma irradiated oocysts in saline solution	I.V.
			286	2.5×10^6 gamma irradiated oocysts in saline solution	I.V.

FCA - Freund's complete adjuvant FIA - Freund's incomplete adjuvant
I.P. - intraperitoneal I.V. - intravenous

Sonicated oocysts were prepared by sonicating a heat killed (80°C for 10 minutes) oocysts suspension until no intact oocysts were visible using light microscopy.

The fusion of mouse 2 resulted in a clone (Cry26) that produced antibody specific to oocyst outer walls (similar to commercially available antibodies to *Cryptosporidium* oocysts). The fusion of mouse 1 spleen cells resulted in three clones (Cry4, Cry5 and Cry6) that produced an antibody that reacted with an oocyst internal antigen. A number of other clones produced antibodies that also recognised oocyst internal antigens. Figures 1 and 2 represents flow cytometric analysis of oocysts and excysted oocysts reacted against a selection of the antibodies. All antibodies except for Cry26 produced more highly fluorescent particles when reacted with excysted oocysts than

with non-excysted oocysts. This demonstrates that the antigens, recognised by these antibodies, are only accessible in open oocysts and are therefore internal.

Oocysts 10^5 (existed or fresh) were aliquotted into each well of a 96 well plate, and 100 µl hybridoma super supernatant added plus 10 µl of FITC-coupled sheep anti-mouse antibody (1/40 dilution) Silenus. Samples were incubated at 37^0C for 30 minutes, then mixed with 200 µl of phosphate buffered saline and analysed by flow cytometry (Vesey, et al, 1994B). The flow cytometer was calibrated with dilutions of a commercially available anti-*Cryptosporidium* antibody so that positive and negative controls were defined.

Analysis of all 41 clones by ELISA, indirect immuno-fluorescence and Western blot produced the results presented in Table 2. All antibodies were positive by ELISA, with results ranging from weakly positive (+) to strongly positive (++++). A range of antigen sites were identified by immunofluorescence including sporozoites, oocyst walls and the interior of oocysts. A series of different antigen-binding patterns were identified by Western blot analysis.

Western blots of *Cryptosporidium parvum* antigens were probed with hybridoma supernatants containing monoclonal antibodies. Solubilised intact oocyst proteins were separated by SDS-polyaccrylamide gel electrophoresis under reducing conditions. Each lane consisted of 1×10^6 oocysts and detection of bound antibodies was by HRP-conjugated anti-mouse antibodies and 4-Chloro-1-napthol. A series of different antigen-binding patterns were identified by Western blot analysis. Each group of antibodies that reacted to a particular antigen on a given site on the oocyst (for example interior, surface, wall, sporozoite) had a characteristc protein binding pattern.

Antibodies Cry26 and Cry4 were purified using EZ-Sep (Amrad Phamacia Biotech, Boronia. Australia) according to the manufacturer's instructions. Purified Cry26 antibody was conjugated with CY3 (Biological Detection Systems, PA, USA) according to the manufacturer's instructions.

Table 2. Characteristics of monoclonal antibodies generated against *Cryptosporidium parvum* oocysts

Mabs	Isotype	ELISA	IFAT
Cry1	M	++++	ND**
Cry2	M	+	ND
Cry3	M	+	ND
Cry4	M	++	oocyst interior
Cry5	M	+++	oocyst interior
Cry6	M	++	oocyst interior
Cry7	M	+++	sporozoite surface
Cry8	ND	+++	sporozoite surface
Cry9	M	+++	sporozoite surface
Cry10	M	++++	ND
Cry11	M	++	ND
Cry12	M	++++	ND
Cry13	M	++	ND
Cry14	M	+++	ND
Cry15	ND	++++	ND
Cry16	M	++	sporozoite surface
Cry17	M	++++	ND
Cry18	M	+++	ND
Cry19	M	+++	sporozoite surface
Cry20	M	+++	sporozoite surface
Cry21	M	++++	ND
Cry22	M	+	ND
Cry23	M	+++	ND
Cry24	M	++	sporozoite surface
Cry25	M	++	oocyst interior
Cry26	M	+++	oocyst wall
Cry27	M	++	sporozoite surface
Cry28	ND	++	sporozoite
Cry29	M	+	sporozoite surface
Cry30	ND	++	sporozoite surface
Cry31	M	++	oocyst wall
Cry32	ND	++	oocyst interior
Cry33	M	++++	sporozoite surface
Cry34	M	++	sporozoite
Cry35	ND	++++	sporozoite
Cry36	M	++++	sporozoite surface
Cry37	ND	+	oocyst
Cry38	M	++++	sporozoite surface
Cry39	M	++++	ND
Cry40	M	++	ND
Cry41	M	++++	sporozoite surface

* IFAT, Indirect Fluorescence Antibody Test

** ND, Not Detected

Oocyst preparation. Oocysts (1×10^8) were surfaced sterilised by suspending in 1 ml of 70% (v/v) ethanol for 5 min and then washing by centrifuging at 13000g for 2 min, discarding the supernatant and resuspending in phosphate buffered saline (PBS), pH 7.4. Excystation was then performed by suspending in 1 ml of acidified PBS, pH 2.75, incubating for 30 min at 37°C, then washing by centrifuging at 13000g for 2 min and resuspending in PBS, pH 7.4 with 0.1% (w/v) sodium deoxycholate and 0.22% sodium hydrogen carbonate. After a further 30 min incubation at 37°C the sample was centrifuged at 13000g for 5 min and fixed by resuspension in 1 ml of PBS, pH 7.4 with 1% (v/v) formalin (excysted oocyst suspension).

Staining pure oocysts. An aliquot (10 µl -10^6 oocysts) of excysted oocyst suspension was mixed with 200 µl of Cry4 (approximately 0.005 mg/ml) and incubated at 37°C for 10 min prior to the addition of 5 µl of a goat anti-mouse FITC conjugated antibody (Silenus Laboratory, Melbourne, Australia; Product DDAF). After a further 10 min incubation at 37°C the sample was analysed by flow cytometry.

Staining seeded environmental samples. An aliquot (10 µl) of excysted oocyst suspension that had been stained with Cry4 as above was mixed with 500 µl (equivalent to 5 litres unconcentrated sample) of a river water sample that had been concentrated by flocculation (Vesey *et al* 1994A) and fixed in 4% (v/v) formalin.

Filtered (0.22 µm) bovine serum albumin fraction V was then added to a final concentration of 2% (w/v) before the addition of 20 µl of CY3 conjugated Cry26 antibody (approximately 0.055 mg/ml). The sample was incubated at 37° for 10 min and then analysed by flow cytometry.

Flow cytometry analysis. A coulter Elite flow cytometer (Coulter Corporation, Miami, USA) and Becton Dickinson Facscan flow cytometer (Becton Dickinson, San Francisco, USA) were used to analyse samples as described previously (Vesey *et al* 1994B). Sorted samples were further examined using epifluorescence microscopy.

RESULTS

Flow cytometric analysis of pure excysted oocysts labelled with Cry4 and FITC resulted in four distinct populations (1, 2, 3 and 4) (Figure 3). Analysis of the sample prior to excystation resulted in only two unlabelled populations labelled (population 1 and 4) (Figure 3). Population 3 reacts strongly with the antibody Cry4, population 2 reacts weakly whereas populations 1 and 4 do not react with Cry4. Examination of the samples using microscopy revealed that the brightly fluorescing population (population 3) is completely excysted empty oocysts and the weakly fluorescing population (population 2) is partially excysted oocysts. The two non-fluorescent populations 1 and 4 were observed to be full oocysts and empty oocysts. The higher light scatter of population 4 indicates that this population represents the full oocysts. Therefore population 1 must represent the empty oocysts.

The lack of populations 2 and 3 in the sample that was not excysted indicates that the antigen recognised by Cry4 is only present in recently excysted oocysts.

Fig. 3 gives a pattern that is characteristic of antibodies that react specifically with recently excysted *Cryptosporidium* oocysts. It will be appreciated that the flow cytometric analysis as described can be used to determine whether any particular antibody reacts to recently excysted *Cryptosporidium* oocysts as defined herein.

Examination of the samples with microscopy revealed that the pre-excystation sample contained 26% empty oocysts, the post-excystation sample contained 53% empty oocysts. This indicates that 27% of the oocysts in the original sample were viable and capable of complete excystation. The percentage of oocysts in the excysted sample that appeared in population 3 was 29%. This confirms that population 3 represents freshly excysted oocysts. The partially excysted oocysts represented by population 2 are viable oocysts that are potentially infections. This type of population has not previously been recognised by standard methods presently in use. The present results therefore demonstrate that by treating oocysts to an excystation procedure prior to staining with Cry4 it is possible to determine oocyst viability.

It was often found that if oocysts were excysted without surface sterilising with ethanol then no reaction with Cry4 could be detected, even though the number of empty oocysts increased after excystation. High

numbers of motile bacteria were observed in these samples, whereas no motile bacteria were observed in samples that had been treated with ethanol.

Results of the analysis of environmental water samples that had been seeded with oocysts stained with both the Cry26 and Cry4 antibodies are presented in Figure 4. The first graph represents side scatter versus red fluorescence (ie, the fluorescence due to binding of CY3 labelled Cry26 and the second graph represents side scatter versus green fluorescence (ie, the fluorescence due to binding of FITC labelled Cry4). A box was drawn on the first graph around an area containing the stained oocysts. This box was then used to gate graph 2 (ie, the only particles that appear on graph 2 are those that appeared in the box). Two distinct populations are present on graph 2, highly fluorescent viable oocysts and non-fluorescent, non-viable oocysts and debris. The viable oocysts are completely separated from all other particles, thus allowing enumeration.

Cryptosporidium oocysts are surrounded by an extremely robust oocyst wall that can protect the organism in the environment. When a viable oocyst is exposed to a temperature of 37°C in acidic solution, followed by an alkaline solution, the sporozoites rapidly break out of the oocysts wall and swim away leaving behind an empty oocyst (Current 1986).

The monoclonal antibody Cry4 recognises an internal antigen in empty *Cryptosporidium* oocysts. The antigen is not accessible in oocysts that have not excysted nor is it present in oocysts that have excysted prior to the excystation treatment. Furthermore, the antigen recognised by Cry4 is removed if oocysts are excysted in the presence of bacteria. This would indicate that the antigen is destroyed by bacterial enzymes.

Previously, oocyst viability has been determined on pure samples of oocysts by performing excystation and then manually counting the number of full and empty oocysts. This method is tedious and labour intensive. The development of the antibody Cry4 or other antibodies that react specifically with recently excysted oocysts will enable immunofluorescence assays employing flow cytometry or other automated technologies to replace this manual methodology.

Methods for determining the viability of small numbers of *Cryptosporidium* oocysts based on the uptake or exclusion of the fluorogenic vital dyes propridium iodide (PI) and 4'6-diamidino-2-phenylindole (DAPI) have been reported (Campbell *et al* 1992). The authors report that dead

oocysts take up PI and fluoresce red, whereas live oocysts exclude PI but are permeable to DAPI resulting in the sporozoites within the oocysts fluorescing blue. The method has proven useful for viability studies on pure oocysts (Robertson *et al* 1992).The application of the method to the routine monitoring of *Cryptosporidium* oocysts in water, however, has been limited due to problems with incorporating the technique into flow cytometric detection methods. The present inventors have experienced problems with the method when analysing turbid environmental samples. In particular, the demand for DAPI by some of the particulate matter present in these samples is higher than the demand for DAPI by the oocysts. This results in the particulate matter staining bright blue and the oocysts not staining. Increasing the concentration of DAPI to ten times that recommended by Campbell *et al* (1992) results in fluorescent oocysts but the fluorescence of the background becomes unacceptably high. Furthermore, the stains PI and DAPI are not specific to oocysts, they may stain any particle that contains DNA. Therefore, these stains cannot be used as specific labels to enable detection with an analysis-only flow cytometer.

The use of the Cry4 antibody to stain samples after they have been excysted enable the routine detection of viable *Cryptosporidium* oocysts in environmental samples. The present inventors have found that the antigen recognised by Cry4 rapidly degrades in samples containing bacterial activity. Therefore, oocysts which have excysted in the environment prior to sample collection will no longer contain the antigen recognised by Cry4.

The recent development of a flow cytometric detection method has solved many of the problems associated with the routine monitoring of *Cryptosporidium* oocysts in water (Vesey *et al* 1993, 1994A). Water utilities are now able to process significant numbers of samples whilst achieving high sensitivities. Unfortunately, the application of the method has been limited by the high cost and sophistication of the flow cytometer. At present, a flow cytometer which can sort is required for the detection of oocysts in water. It has been shown, however, that a simple and less expensive analysis-only cytometer is capable of detecting a single specific microorganism in turbid water samples when the organism is labelled with two specific probes (Vesey *et al* 1994B), such as antibodies. Until now it has not been possible to label *Cryptosporidium* oocysts with more than one antibody because all commercially available antibodies recognise the same epitope on the surface of

the oocyst wall (Moore *et al* 1995). By performing excystation and then staining with an antibody which reacts specifically to recently excysted oocysts and a surface antibody according to on method of the present invention it is now possible to dual label oocysts and detect them using a simple analysis-only flow cytometer.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

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Claims

1. A method of detecting the presence of viable *Cryptosporidium* oocysts in a sample containing *Cryptosporidium* oocysts, the method comprising the steps of:

a) treating the sample so as to cause any viable oocysts of *Cryptosporidium* in the sample to excyst;

b) exposing the treated sample to an antibody that binds specifically to recently excysted *Cryptosporidium* oocysts such that the antibody binds to recently excysted *Cryptosporidium* oocysts in the sample; and

c) detecting the presence of oocyst-bound antibody in the sample.

2. The method according to claim 1 such that the *Cryptosporidium* is *Cryptosporidium parvum*.

3. The method according to claim 1 or 2 such that the oocysts are caused to excyst by incubating the sample at about 37°C under acidic conditions, followed by incubating the sample under neutral to alkaline conditions at about 37°C.

4. The method of claim 3 wherein the oocysts are caused to excyst by incubating the sample at 37°C at pH 2 to 4 for 10 to 60 minutes, followed by incubating the sample at 37°C at pH 7 to 9 for 10 to 60 minutes.

5. The method of claim 4 wherein the oocysts are caused to excyst by incubating the sample at 37°C at pH 2.75 for 30 minutes, followed by incubating the sample at 37°C at pH 7.4 for 30 minutes.

6. The method according to any one of claims 1 to 5 wherein the recently excysted *Cryptosporidium* oocysts are excysted oocysts in the sample up to about one hour after the treatment of step (a).

7. The method according to any one of claims 1 to 6 such that the antibody is fluorescently labelled prior to use and the binding of the antibody to the recently excysted oocysts is detected by measuring directly the fluorescence of the oocyte-bound antibody.

8. The method according to any one of claims 1 to 7 such that the binding of the antibody to the recently excysted oocysts is measured indirectly by further treating the sample with a fluorescently-labelled ligand that binds specifically to the antibody and measuring the binding of the labelled ligand to the oocyte-bound antibody.

9. The method according to any one of claims 1 to 8 such that the sample is analysed by flow cytometry or microscopy to detect the oocyst-bound antibody.

10. The method according to any one of claims 1 to 9 such that the antibody is a monoclonal antibody.

11. The method according to claim 10 such that the monoclonal antibody is selected from the group consisting of Cry4, Cry5 and Cry6.

12. The method according to claim 11 such that the monoclonal antibody is Cry4.

13. The method according to any one of claims 1 to 12 such that the excysted oocysts are exposed to a first antibody that binds specifically to recently excysted oocysts and a second antibody that binds specifically to the surface of *Cryptosporidium* oocysts.

14. The method according to claim 13 such that the first and second antibodies are labelled with different fluorescent labels such that antibody binding is detected by measuring the respective fluorescence of each fluorescent label with a simple analysis-only flow cytometer.

15. The method according to claim 14 such that the second antibody binds to both viable and non-viable oocysts and the detection of the binding of one or more of the antibodies to the oocysts is used to indicate the presence *Cryptosporidium* oocysts in the sample.

16. The method according to claim 15 such that the first antibody is Cry4 and the second antibody is Cry26.

17. An antibody that binds specifically to recently excysted oocysts of *Cryptosporidium*.

18. The antibody according to claim 17 being the monoclonal antibody is selected from the group consisting of Cry4, Cry5 and Cry6.

19. An hybridoma cell producing a monoclonal antibody that binds specifically to recently excysted oocysts of *Cryptosporidium*.

20. The hybridoma cell which produces the monoclonal antibody selected from the group consisting of Cry4, Cry5 and Cry6.

21. A ligand or ligands of recently excysted oocysts of *Cryptosporidium* that is specifically bound by the monoclonal antibody Cry4, Cry5 or Cry6.

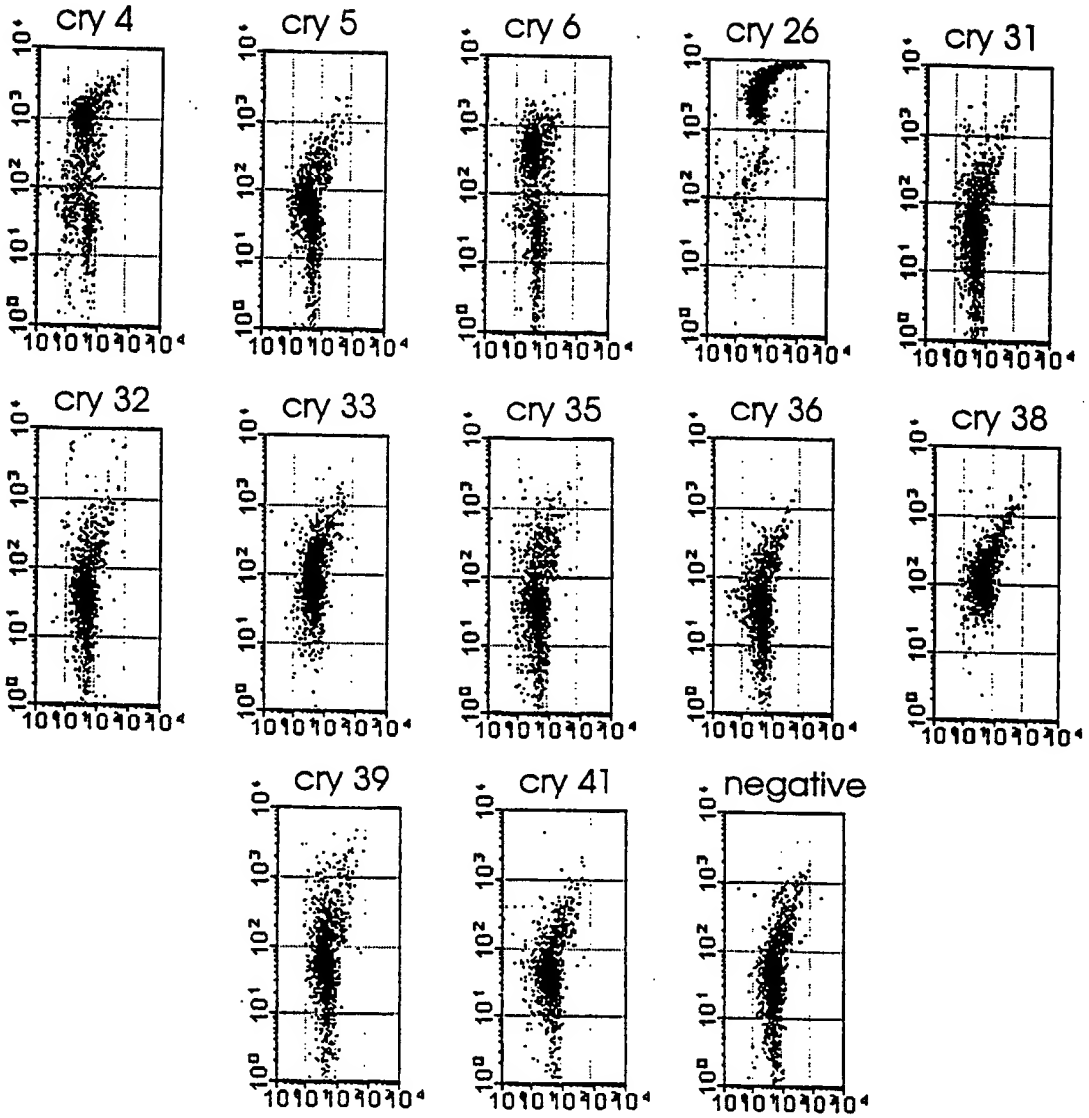
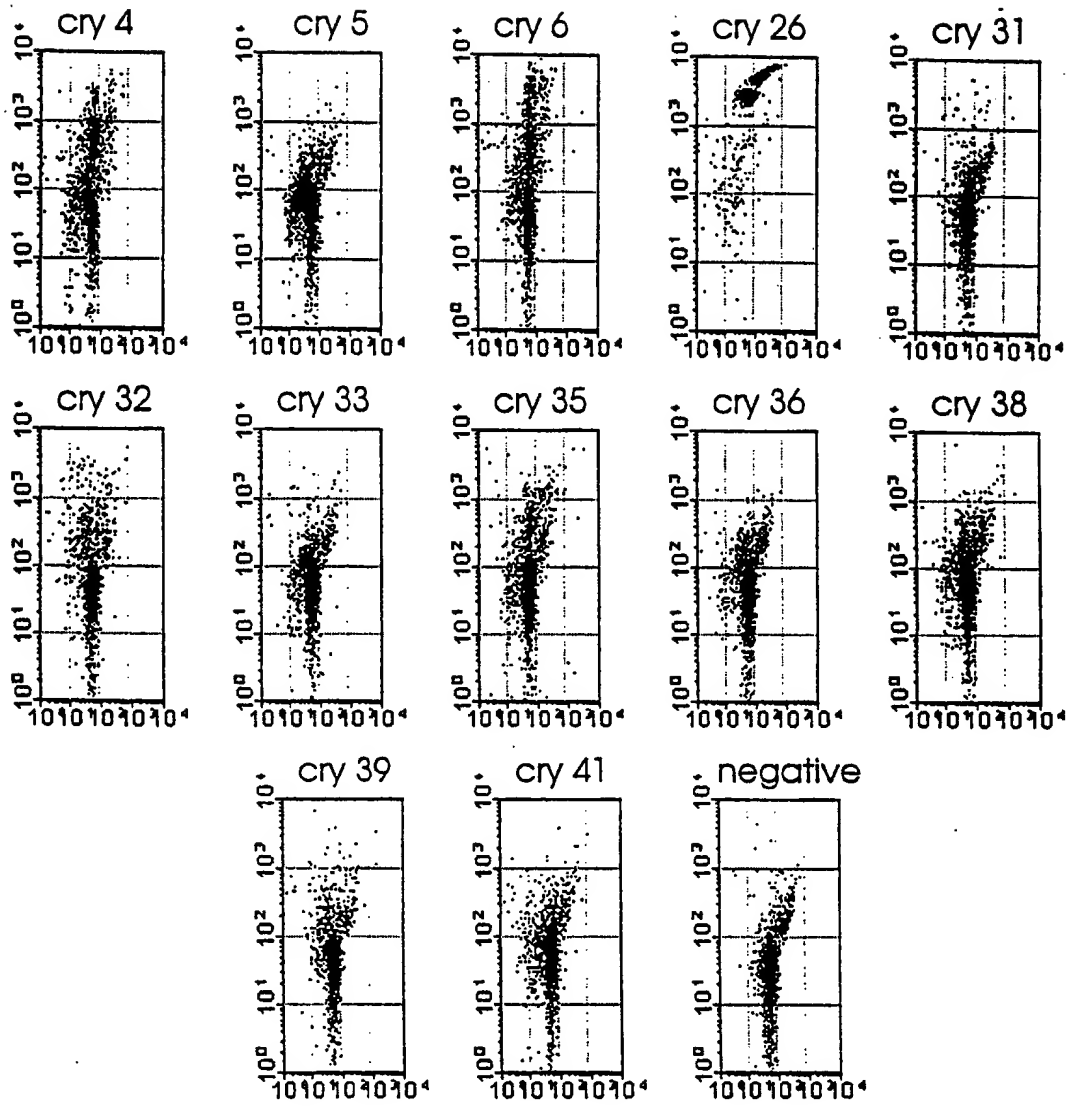

cry 4
cry 5
cry 6
cry 26
cry 31
cry 32
cry 33
cry 35
cry 36
cry 38
cry 39
cry 41
negative

Fig. 1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cry 5
cry 6
cry 26
cry 31
cry 32
cry 33
cry 35
cry 36
cry 38
cry 39
cry 41
negative

Fig. 2

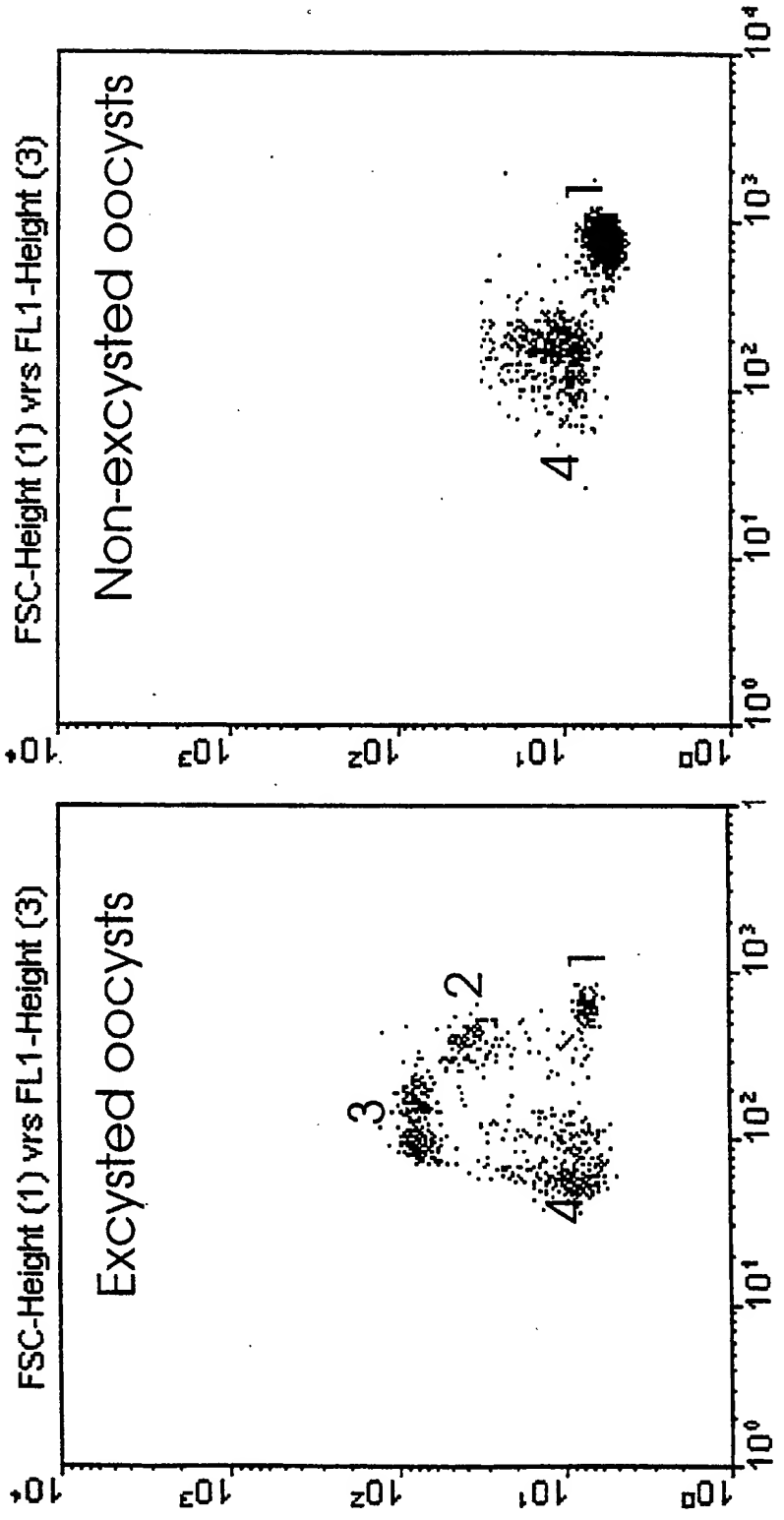
FSC-Height (1) vrs FL1-Height (3)
Non-excysted oocysts
1
4
FSC-Height (1) vrs FL1-Height (3)
Excysted oocysts
1
2
3
4

Fig. 3

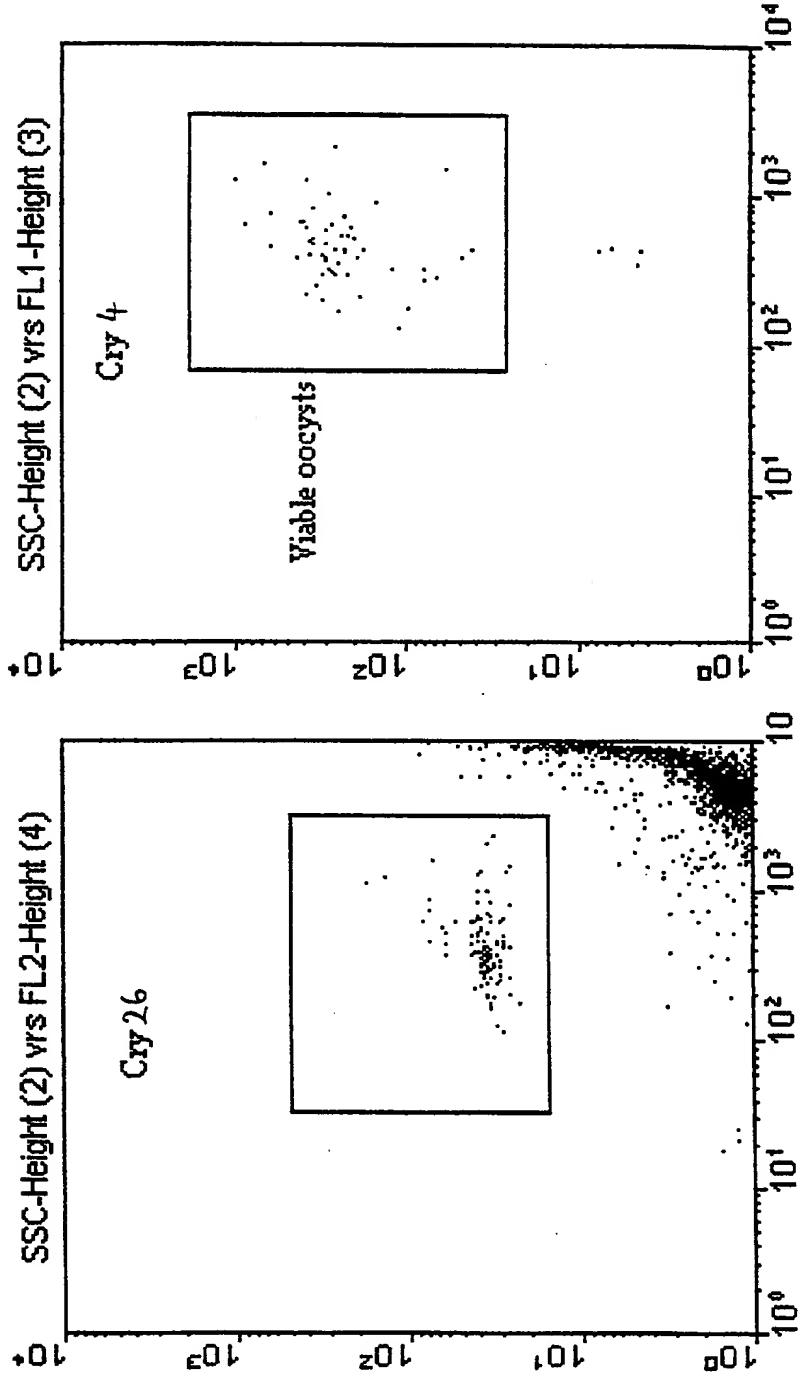
SSC-Height (2) vrs FL1-Height (3)
Cry 4
Viable oocysts
SSC-Height (2) vrs FL2-Height (4)
Cry 26
10⁰
10¹
10²
10³
10⁴

Fig. 4

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU96/00543

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C07K 16/20 G01N 33/569 33/577

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
WPAT, CHEMICAL ABSTRACTS (See Keywords in the electronic database box below)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
JAPIO, USPM, MEDLINE (See Keywords in the electronic database box below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT, JAPIO, USPM, KEYWORDS: CRYPTOSPORIDI: and OOCYST#
MEDLINE, CHEMICAL ABSTRACTS, KEYWORDS: CRYPTOSPORIDI? and ANTIBOD? and OOCYST?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93/24649 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 9 December 1993. See for example page 7 lines 31-37 examples 14 & 15.	17, 19
Y		1, 2, 6-10
P X	JOURNAL OF EUKARYOTIC MICROBIOLOGY (1995 July - Aug) 42(4) 395-401 BONNIN A et al. "Monoclonal antibodies identify a subject of dense granules in Cryptosporidium parvum zoites and gamonts"	17, 19
P Y	See especially page 395 and page 400.	1, 2, 6-10

☐ Further documents are listed in the continuation of Box C ☐ See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 December 1996	13.12.96
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (06) 285 3929	Authorized officer J.H. CHAN Telephone No.: (06) 283 2340

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU96/00543

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VETERINARY PARASITOLOGY 53 (1994) 151-166 Oretga - Mara L.M. et al "Identification of Cryptosporidium parvum oocyst/sporozoite antigens recognized by infected and hyperimmune lambs" See for example page 160.	17, 19
X	INFECTION AND IMMUNITY May 1994 page 1927-1939 Riggs M. W. et al "Bovine antibody against Cryptosporidium parvum elicits and circumsporozoite precipitate-like reaction and has immunotherapeutic effect against persistent Cryptosporidiosis in SCID mice" See for example page 1937.	17, 19
Y		1, 2, 6-10
X	PARASITOLOGY (1995) 110 page 259-268 McDonald V. et al "Localization of parasite antigens in Cryptosporidium parvum - infected epithelial cell using monoclonal antibodies" See for example page 265.	17, 19
Y		1, 2, 6-10
X	PARASITOLOGY RESEARCH 1993 79:8-14 Bonin A et al "A new antigen of Cryptosporidium parvum micronemes possessing epitopes cross-reactive with macrogaemete granules" See pages 8, 9 and 13.	17, 19
Y		1, 2, 6-10
X	INFECTION AND IMMUNITY June 1992 page 2343-2348 Petersen C et al "Identification and initial characterization of five Cryptosporidium parvum sporozite antigens genes" See for example page 2344.	17, 19
Y		1, 2, 6-10
X	INFECTION AND IMMUNITY May 1991 page 1703-1708 Bonin A et al "Characterization of microneme antigens of Cryptosporidium parvum (Protoza, Apicmplexa)" See page 1703.	17, 19

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No.
PCT/AU96/00543

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member			
WO	93/24649	AU	44096/93	NZ	253620

END OF ANNEX